In vitro antimycobacterial activity of six Cameroonian medicinal plants using microplate alamarBlue assay

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Objective/background: The latest incidence of tuberculosis (TB) (per 100,000 people) in Cameroon was 243.00 as of 2011. Over the past 21 years, the value for this indicator has fluctuated between 112.00 in 1990 and 320.00 in 2003. Worldwide, this incidence has also increased, bringing back TB as a reemerging disease. On the same note, resistance to anti-TB drugs has increased, urging the search for new molecules.

Methods: This study was carried out to evaluate the antimycobacterial activity of six medicinal plants on the virulent strain, H37Rv, using the microplate alamarBlue assay. Mycobacterium tuberculosis (H37Rv strain) was incubated with decreased concentrations of six plant extracts, ranging from 250 μg/mL to 31.25 μg/mL. After 7 days of incubation at 37 °C, the effects of these plant extracts on the viability of the mycobacteria were evaluated. For each plant extract, the minimal inhibitory concentration was determined.

Results: The results showed that the compounds MBC1, MBC24, MBC68, MBC81, MBC117, and MBC118 were the best candidates with minimal inhibitory concentrations of 31.25, 62.5, 125, 62.5, and 125 μg/mL, respectively.

Conclusion: These results confirm and validate the traditional use of these plants to treat respiratory diseases, which could be good sources and alternatives of plant metabolites for anti-TB drug development.

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within communities [3–5]. While TB itself has historically been of central concern of public health and infectious diseases, the last few decades have witnessed the rise of human immunodeficiency virus/AIDS, an immunosuppressive illness that amplifies the infectivity of the tubercle bacillus and catalyzes its conversion from latent to active infection [6]. Furthermore, the emergence of M. tuberculosis strains resistant to conventional first-line and second-line antitubercular treatment is particularly worrisome [7,8], since no new anti-TB drugs have been introduced into the market since 1967. Moreover, up to 50 million people are infected with drug-resistant forms of TB with about 500,000 cases of multidrug-resistant TB a year worldwide [9]. Even though there are currently new lead compounds being characterized for TB treatment [10,11], they are challenged by poor accessibility, high costs, long treatment regimen, and low adherence owing to the toxicity of second-line drugs. The newly commercialized drug is likely to be exhausted with the emerging resistance, emphasizing the imperative continuous search, identification, and characterization of more compounds for anti-TB drugs.

Medicinal plants have been used for centuries as nonexhaustive sources of metabolites for drug development and as an alternative remedy for treating human diseases, as they contain numerous active constituents of therapeutic value [12–15]. Rational chemistry, while essential to the development of many modern pharmaceuticals, often serves better to refine the chemical blueprints isolated from natural product screens than to devise entirely new molecular backbones. The enormous diversity of plant-derived compounds therefore makes them one of the most promising reservoirs of potentially novel anti-TB molecules. Cameroon is renowned for its rich and uncharacterized biodiversity [16], and Cameroonian medicinal plants are frequently used to treat diseases as a means of reducing reliance on expensive imported and/or chemical drugs. Such plants should be identified and screened on the basis of traditional knowledge for efficacy in the treatment of TB.

This work reports on the in vitro anti-TB evaluation of medicinal plants identified during an ethnobotanical survey carried out in Kala at the Department of "Méfou-et-Akono"/Centre Region and in Dschang at the Menoua Department/West Region. This survey investigated on medicinal plants used by traditional healers to treat cough and pulmonary diseases.

**Materials and methods**

**Plant collection and conditioning**

Plants (bark, roots, and stalk) were collected based on the information obtained from traditional healers from Kala at the Department of "Méfou-et-Akono"/Centre Region and in Dschang at the Menoua Department/West Region of Cameroon. In Cameroon, generally, most people rely on traditional healers for their primary health care. These traditional healers used plants to treat persisting cough and chest pain. They have inherited the knowledge from their parents, grandparents, and ancestors.

The collected plants were identified at the National Herbarium of Yaoundé, where voucher-specimen numbers were obtained. Figs. 1 and 2 are photographs of two of the plants used (Garcinia preussii and Acanthus montanus). The plant material was dried and ground into coarse or fine powder depending on their texture.

**Preparation of plant extract**

Crude extracts of plant parts were prepared as follows:

1. For hot extraction, 40 g of the powdered bark or stalk was weighed and put into the extraction thimble on the Soxhlet to which 250 mL of the solvent was added. The various solvents used were methanol, hexane, and ethyl acetate. The Soxhlet was switched on and extraction was carried out for 2 h and 30 min. The solvent was removed from the extract by evaporation on a rotavapor.
2. For cold extraction, 100 g of powdered roots or stalk was weighed and added into 500 mL of the solvent. Extraction was carried out at room temperature with frequent shaking for 48 h. The filtrates were evaporated to dryness using the rotavapor.

The obtained crude extracts were thus stored at 4 °C for subsequent work. The phytochemical screening of the plant extracts was carried out by the method described by Harborne [17].

**In vitro anti-TB screening**

*M. tuberculosis* culture preparation

The *M. tuberculosis* reference strain, H37Rv, was used. Ten milliliters of Middlebrook 7H9 broth supplemented with 10% oleic acid–albumin–dextrose–catalase and 0.2% of glycerol culture was inoculated with 0.4–0.6 mL of freeze stock of H37Rv in a 50 mL conical tube. The culture was grown to mid-log phase on the wheel at 37 °C, until OD600 = 0.4–0.8. Using 7H9 broth without Tween 80, the culture was diluted to OD600 = 0.001. This resulted in a culture with approximately 10⁵ CFU/mL. Then, 100 μL of this culture was used to set up the assay plates, with each well containing 10⁴ CFU.

Antimycobacterial-activity tests

The antimycobacterial activity of the plant crude extracts was tested using the microplate alamarBlue assay [18,19].

![Fig. 1 – Leaves of Garcinia preussii.](Image)
The susceptibility test was done in 96 microtiter plates using the alamarBlue reagent as an indicator of cellular viability. Working solutions of the tested extracts were diluted in Middlebrook 7H9 broth supplemented with oleic acid–albumin–dextrose–catalase to obtain the final sample concentrations that ranged from 250 \( \mu g/mL \) to 31.25 \( \mu g/mL \). Isoniazid was dissolved in dimethyl sulfoxide and used as a positive control drug at 1.28 \( \mu g/mL \) as the starting concentration, and extracts/drug-free medium with strain suspensions were used as the negative control. One hundred microliter of 7H9 broth were added into all wells of the 96-well plate, and 100 \( \mu L \) of the compounds/extracts was introduced to the wells in the first row (A) and mixed thoroughly. The sample mixture (100 \( \mu L \)) was removed from wells of row A to perform a twofold serial dilution down the rows (B–H). The last 100 \( \mu L \) was discarded. Then, 100 \( \mu L \) of the inoculum was introduced into the corresponding wells. The final volume in each well was 200 \( \mu L \). Each extract concentration was assayed in duplicate. Each microplate was then sealed with the optical sealing tape and incubated for 7 days at 37°C in normal atmosphere. After the incubation period, 32.5 \( \mu L \) of alamarBlue was added to each well. The plates were then reincubated for 16–18 h at 37 °C in the dark. The experimental results were computerized using the BMG, Leicester, United Kingdom OPTIMA microplate reader at 544ex/590em for data analysis.

The minimal-inhibitory-concentration (MIC) results were presented as mean value. The lowest concentration that resulted to 90% inhibition was defined as the MIC. The MIC values determined by this method were cross-checked using the broth-dilution methods. A blue color in the well was scored as “no mycobacterial growth,” and a pink color was scored as “growth occurrence” \[20,21\].

**Results**

**Plants collected**

Table 1 presents the identification of the six studied plants, by scientific and family names, traditional usage, and voucher-specimen number. These plants belong to the following families: Annonaceae, Vitaceae, Rubiaceae, Urticaceae, Lauraceae, and Acanthaceae.

**Crude-extract preparation**

Six different crude extracts were prepared with different solvents of extraction. The plant parts analyzed, the solvents used, and the yields of extraction are presented in Table 2.

**Antimycobacterial activity**

The antimycobacterial activity of the plant crude extracts has been evaluated on the virulent strain H37Rv at the highest concentration of 250 \( \mu g/mL \). The six tested extracts, namely, A. montanus, Beilschmiedia obscura, Cissus petiolata, Enantia chlorantha, Urera repens, and Garcinia preussii, were active against M. tuberculosis with MICs ranging from 31.25 \( \mu g/mL \) to 250 \( \mu g/mL \). These results are summarized in Table 3.

The most active anti-TB effect was obtained from the methanolic extract of B. obscura with an MIC of 31.25 \( \mu g/mL \) that inhibits the growth of M. tuberculosis at 96.2%. The methanolic extract of A. montanus and U. repens each exhibited an antimycobacterial activity with an MIC of 62.5 \( \mu g/mL \) and a percentage of growth inhibition of 95.06% and 98.4%, respectively.

**Phytochemical screening of the plant extracts**

The screens identified various compounds from the plant extracts. These compounds include phenols, sterols, saponins, flavonoids, and glycosides. These results are presented in Table 4 for each plant.

**Discussion**

Beilschmiedia species are known to produce many types of phytochemicals \[22–24\] with various biological activities. Besides, Fankam, Kuiate, and Kuete \[25\] worked on fruits of B. obscura, and found that they were highly active against a panel of Gram-negative bacteria.

Plants from the Acanthaceae family are widely used traditionally for the treatment of various ailments, such as infectious diseases \[26,27\]. The antimycobacterial activity observed from Acanthus montanus corroborated with the study of Ikezu, Ajiwe, Ilozue, and Chukwukanne \[28\], who worked on the leaves of A. montanus. They found that, in comparison with the activity of some standard antibiotics, the leaves of A. montanus were more active against Gram-negative and Gram-positive bacteria.

The roots of G. preussii displayed an MIC of 125 \( \mu g/mL \) on the virulent strain H37Rv. The biological activity of compounds
isolated from Garcinia on Escherichia coli, Pseudomonas aerugi-
nosa, Staphylococcus aureus, and Enterococcus faecalis has
already been evaluated and shows interesting activities [29].
Kaikabo and Eloff [30] isolated two biflavonoids from Garcia-
in, and found that they were active against fast-growing and
nonpathogenic Mycobacterium smegmatis, and had a good activ-
ity against nosocomial bacteria.
Although no antibacterial activity has yet been evaluated
on C. petiolata, a plant belonging to the Vitaceae family,
Garima, Saurabh, and Nagori [31] did an overview on the

Table 1 – Plant name, family name, and traditional use of collected plants.

<table>
<thead>
<tr>
<th>Plant code</th>
<th>Scientific name</th>
<th>Family name</th>
<th>Traditional usage</th>
<th>Voucher-specimen number</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBC1</td>
<td>Enantia chlorantha</td>
<td>Annonaceae</td>
<td>Malaria, body pains, gastrointestinal troubles, cough</td>
<td>28724/SRF/Cam</td>
</tr>
<tr>
<td>MBC17</td>
<td>Cissus petiolata</td>
<td>Vitaceae</td>
<td>Asthma, cough, hemorrhoids, gonorrhea</td>
<td>9163 SRF Cam</td>
</tr>
<tr>
<td>MBC24</td>
<td>Beilschmiedia obscura</td>
<td>Lauraceae</td>
<td>Friction on localized pains, respiratory problems</td>
<td>1004/SRFK</td>
</tr>
<tr>
<td>MBC68</td>
<td>Urra repens</td>
<td>Urticaceae</td>
<td>Abscess, headache, purge, asthma</td>
<td>7450/SRF/Cam</td>
</tr>
<tr>
<td>MBC117</td>
<td>Acanthus montanus</td>
<td>Acanthaceae</td>
<td>Fever, furuncles, cancer, ulcer, cough</td>
<td>2127/SRFK</td>
</tr>
<tr>
<td>MBC118</td>
<td>Garcinia preussei</td>
<td>Clusiaceae</td>
<td>Stomachsches, toothaches, chewstick, cough</td>
<td>19325/SRF/Cam</td>
</tr>
</tbody>
</table>

Table 2 – Solvents used and yield of extraction.

<table>
<thead>
<tr>
<th>Plant code</th>
<th>Scientific name</th>
<th>Part used</th>
<th>Extraction solvent</th>
<th>Yield (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBC1</td>
<td>Enantia chlorantha</td>
<td>Bark</td>
<td>CH₂OH Hot extraction</td>
<td>1.70</td>
</tr>
<tr>
<td>MBC17</td>
<td>Cissus petiolata</td>
<td>Stalk</td>
<td>CH₂OH Hot extraction</td>
<td>0.24</td>
</tr>
<tr>
<td>MBC24</td>
<td>Beilschmiedia obscura</td>
<td>Roots</td>
<td>CH₃COOC₂H₅ Cold extraction</td>
<td>0.70</td>
</tr>
<tr>
<td>MBC68</td>
<td>Urra repens</td>
<td>Stalk</td>
<td>CH₂OH Hot extraction</td>
<td>0.66</td>
</tr>
<tr>
<td>MBC117</td>
<td>Acanthus montanus</td>
<td>Stalk</td>
<td>Hexane</td>
<td>0.72</td>
</tr>
<tr>
<td>MBC118</td>
<td>Garcinia preussei</td>
<td>Roots</td>
<td>Hexane/ethyl acetate 50:50</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 3 – Antimycobacterial activity of the six plant extracts.

<table>
<thead>
<tr>
<th>Plant code</th>
<th>Name of the plant</th>
<th>Part used</th>
<th>MIC (µg/mL)</th>
<th>Percentage of inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBC1</td>
<td>Enantia chlorantha</td>
<td>Bark</td>
<td>250</td>
<td>91.9</td>
</tr>
<tr>
<td>MBC17</td>
<td>Cissus petiolata</td>
<td>Stalk</td>
<td>250</td>
<td>97.9</td>
</tr>
<tr>
<td>MBC24</td>
<td>Beilschmiedia obscura</td>
<td>Roots</td>
<td>31.25</td>
<td>96.2</td>
</tr>
<tr>
<td>MBC68</td>
<td>Urra repens</td>
<td>Stalk</td>
<td>62.5</td>
<td>98.4</td>
</tr>
<tr>
<td>MBC117</td>
<td>Acanthus montanus</td>
<td>Stalk</td>
<td>62.5</td>
<td>95.06</td>
</tr>
<tr>
<td>MBC118</td>
<td>Garcinia preussei</td>
<td>Roots</td>
<td>125</td>
<td>96.7</td>
</tr>
</tbody>
</table>

MIC = minimal inhibitory concentration.

Table 4 – Results of the phytochemical tests done on four of the six plant extracts.

<table>
<thead>
<tr>
<th></th>
<th>Beilschmiedia obscura</th>
<th>Urra repens</th>
<th>Acanthus montanus</th>
<th>Garcinia preussei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenols</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Triterpenes</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sterols</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lipids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Sugars</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

The symbol “+” means “present,” while the symbol “–” means “absent.”
pharmacological and therapeutic activity of *Cissus quadrangularis*, a plant from the same family. They reported that two asymmetrical tetracyclic triterpenoids and calcium were identified as its major constituents, and that it possesses antibacterial and antifungal activities [32–35]. Moreover, the phytochemical study of the aerial part of *C. quadrangularis* done by Ruskin et al. [15] showed the presence of alkaloids, tannins, and flavonoids. These wide variety of phytochemical compounds could justify the antimycobacterial activity observed with *C. petiolarata*.

The investigation of *E. chlorantha* stem barks showed that they contained a large quantity of phenols, alkaloids, sapo-

It is worth pointing out that the activities showed from the plants studied are those from crude and unpurified, thus non-concentrated, compounds. It is expected that fractionation of these crude extracts will improve the MIC observed.

**Conclusion**

The obtained results confirm and validate the traditional use of some of these plants, which could be good sources and alternative of metabolites for anti-TB-drug development. These encouraging results prompted us to pursue the evaluation of the most active extracts. Therefore, fractionation and further phytochemical and pharmacological studies of these plants are evidently worthy, and our group is focusing on this effort.

**Conflicts of interest**

The authors declare that they have no competing interests.

**Acknowledgments**

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**References**

[7] Center for Disease Control and Prevention, Division of Tuberculosis Elimination, Core curriculum on tuberculosis: what the clinician should know, 1600 Clifton Road, Atlanta, GA, USA, Centers for Diseases Control and Prevention; 2003.


